Immune Responses in Farm Workers after Exposure to *Bacillus thuringiensis* Pesticides

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Although health risks to pesticides containing *Bacillus thuringiensis* (*Bt*) have been minimal, the potential allergenicity of these organisms has not been evaluated. Therefore, a health survey was conducted in farm workers before and after exposure to *Bt* pesticides. Farm workers who picked vegetables that required *Bt* pesticide spraying were evaluated before the initial spraying operation ($n = 48$) and 1 and 4 months after ($n = 32$ and 20, respectively). Two groups of low- ($n = 44$) and medium- ($n = 34$) exposure workers not directly exposed to *Bt* spraying were also assessed. The investigation included questionnaires, nasal/mouth lavages, ventilatory function assessment, and skin tests to indigenous aeroallergens and to a variety of *Bt* spore and vegetative preparations. To authenticate exposure to the organism present in the commercial preparation, isolates from lavage specimens were tested for *Bt* genes by DNA–DNA hybridization. Humoral immunoglobulin G (IgG) and immunoglobulin E (IgE) antibody responses to spore and vegetative *Bt* extracts were assayed. There was no evidence of occupationally related respiratory symptoms. Positive skin-prick tests to several spore extracts were seen chiefly in exposed workers. In particular, there was a significant ($p < 0.05$) increase in the number of positive skin tests to spore extracts 1 and 4 months after exposure to *Bt* spray. The number of positive skin test responses was also significantly higher in high ($p < 0.05$) than in low- or medium-exposure workers. The majority of nasal lavage cultures from exposed workers was positive for the commercial *Bt* organism, as demonstrated by specific molecular genetic probes. Specific IgE antibodies were present in more high-exposure workers ($p < 0.05$) than in the low and medium groups. Specific IgG antibodies occurred more in the high ($p < 0.05$) than in the low-exposure group. Specific IgG and IgE antibodies to vegetative organisms were present in all groups of workers. Exposure to *Bt* sprays may lead to allergic skin sensitization and induction of IgE and IgG antibodies, or both. *Key words: Bacillus thuringiensis*, *Bt* genes, farm workers, IgE sensitization, IgG antibodies, nasal lavage, pesticides. *Environ Health Perspect* 107:575–582 (1999). [Online 7 June 1999] *http://ehpnet1.niehs.nih.gov/docs/1999/107p575-582bernstein/abstract.html*

Microbial pesticides, which were developed to avoid the toxicity associated with many chemical pesticides, have been used for large scale pest eradication for more than 30 years. The chief organism used for this purpose is *Bacillus thuringiensis* (*Bt*). Subspecies of this organism, var. *kurstaki* (*Btk*), var. *aizawai* (*Bta*), and var. *israelensis* (*Bti*) are among the most commonly used strains. More than 1 million pounds of these pesticides are applied annually in the United States alone. *Bt* is a gram-positive, spore-forming bacillus that is distinguished from *B. cereus* and *B. subtilis* by the presence of a parasporal body (PIB) commonly referred to as the toxin crystal (cry) (*1*). The crylike PIB structures contain several polypeptide products, including an abundance of pro-δ-endotoxins and their proteolytically cleaved by-products. Each endotoxin type is encoded by a different cry gene; they are usually synthesized and assembled as part of the PIB structures during sporulation (*1*). Contemporary commercial *Bt* pesticide formulations are complex in composition; they contain large amounts of spores ($> 10^9$/mL of product) in close association with intact and partially assembled and/or degraded PIB crys, residual amounts of fermentation medium, cell wall debris, and trace amounts of vegetative cells (*2*).

Safety assessments of *Bt* have focused primarily on the potential pathogenicity of the organism and toxicity of the cry for mammalian species (*3*). Potential allergic reactions associated with the use of *Bt* have not been considered, although an alkaline protease produced by a related organism, *B. subtilis*, has been identified as a respiratory allergen and studied extensively because of occupational exposure in the detergent industry (*4,5*). Only one documented and three other questionable cases of overt human disease associated with *Bt* pesticide use have been reported (*6*). In this public health survey of a large number of individuals exposed to a massive *Bt* pesticide spraying program, some of the symptoms reported included rash and angioedema (*6*). One of the spray workers in this project developed dermatitis, pruritus, swelling, and erythema with conjunctival injection. *Bt* was cultured from the conjunctivae. In 1992 the use of *Bt* in an Asian gypsy moth control program was associated with classical allergic rhinitis symptoms, exacerbations of asthma, and skin reactions among exposed individuals reporting possible health effects after the spraying operation (*7*). Unfortunately, there was no follow-up to determine whether these events were *Bt*-induced hypersensitivity or toxic reactions or merely due to common aeroallergens coincidental to the season during which the spraying occurred (*8*). Similar findings occurred during another *Bt* spraying in the spring of 1994 (*8*). Allergenicity is of particular concern because approximately 75% of asthma cases are triggered by allergens (*9*) and morbidity and mortality due to asthma have increased considerably over the past 20 years (*10*). Although the evidence thus far does not directly implicate human health risks with the use of *Bt*, it is clear that potential allergenicity of these strains should be evaluated. To accomplish this goal, a surveillance program was conducted in a group of farm workers before and after exposure to *Bt* pesticides. The investigation included detailed questionnaires to assess symptoms associated with allergic syndromes, nasal/mouth lavages to assess exposure, a ventilatory index of lung function, skin tests to common aeroallergens

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and a variety of *Bt* spore preparations, and the collection of sera for humoral antibody tests to *Bt* spore and vegetative extracts. Microbial flora from lavages as well as organisms isolated from various sampling activities before and after spray operations were characterized by several methods.

Methods

Subjects and study design. Volunteers for this investigation were recruited from a group of seasonal farm workers employed as vegetable harvesters in the muck crops region of northern Ohio from June to October 1995. The recruitment process consisted of a preliminary meeting with prospective volunteers. All aspects of the clinical study were explained in English and in Spanish. Second, bilingual orientation was given prior to the volunteers' signatures on informed consents in accordance with the U.S. Environmental Protection Agency common rule for the protection of participants. The Institutional Review Board of the University of Cincinnati (Cincinnati, OH) reviewed and approved this process. Table 1 shows the study design and Table 2 summarizes the demographic data of the workers. Group 1 consisted of 48 workers who picked vegetables (celery, parsley, cabbage, kale, spinach, and strawberries) that required *Bt* pesticide spraying soon after the first crops were planted and continued until the harvesting of the last crop in early October. The workers in this group were evaluated before exposure to the first *Bt* pesticide (Javelin; Sandoz Agro, Inc., Des Plaines, IL) spraying in that particular growth season (visit 1). One month after the initial spraying operation, 32 workers of group 1 returned for another evaluation. They were designated the high-exposure workers (visit 2). Twenty group 1 high-exposure workers who were tested in visits 1 and 2 and exposed to additional *Bt* treatments from June to October were reassessed in early October or 4 months after spraying was begun (visit 3). A group of low *Bt*-exposure workers (group 2), working with a crop (onions) not requiring *Bt* spraying at areas 3 miles away from group 1 workers were also investigated. It was presumed that because of their work locations and the crops that they handled, exposure to *Bt* would be minimal or less than the workers in group 1. Finally, a medium *Bt*-exposure group of workers (group 3), who received the vegetables from the field, washed them, and packed them in wooden crates, was also investigated. These workers handled all vegetables (sprayed and nonsprayed) and therefore could have been exposed to *Btk* either through direct handling or aerosols created during the washing and packing processes. Because the primary evaluation goals of this study were skin test antibody levels and *Bt* spores present in nasal washes, the differences in crops that were handled by groups 1, 2, and 3 were not considered significant. Before participating, all volunteers read and signed a bilingual (English and Spanish) informed consent approved by the University of Cincinnati Institutional Review Board.

At the initial visit a detailed medical/occupational questionnaire was administered by a physician. In addition to past social and medical histories, questions about possible work-related lower and upper respiratory and/or dermatological problems were included. Peak expiratory flow rate testing was performed, followed by nasal/mouth washes, skin tests, and venipuncture for antibody assays.

The questionnaire and peak expiratory flow rate. The questionnaire, which was designed to elicit occupationally related symptoms, had been used by this laboratory in previous occupational cross-sectional and longitudinal studies (*11,12*). The physician reviewer based a case definition of occupational asthma on the presence of at least two lower respiratory symptoms (wheeze, cough, shortness of breath) at work combined with improvement on a work-free day(s) and a latency period between exposure to *Bt* pesticides and onset of symptoms. Similarly, a case definition of occupational rhinitis was established by the presence of at least two upper respiratory symptoms (nasal stuffiness, runny nose, and/or itchy eyes) at work, combined with improvement on a work-free day(s) and a latency period. Diagnosis of occupational dermatitis required the occurrence of at least two skin symptoms (pruritus, hives, skin rash) at work, improvement on a work-free day(s), and a latency period before the initial onset of symptoms. The best of three peak expiratory flow rate efforts was selected, compared to normal values based on age and height, and expressed as a percent of predicted normal (*13*).

Skin tests. To determine atopic status, skin-prick tests to a battery of aeroallergens (short ragweed, blue grass, timothy, a mixture of Ohio trees, dust mite, *Alternaria alternata*) were applied to one of the forearms. Atopy

Table 1. Study design.

Site visit	Group	Purpose	Crops handled	*Bt* exposure	Procedures
1	NA	Prestudy orientation for volunteers	NA	NA	Explanation of study with question and answer session
2	1	Before first *Bt* spray	Celery, parsley, cabbage, kale, spinach, strawberries	NA	Questionnaire, PEFR, lavages, skin tests, blood
3	1	One month after first *Bt* spray	Same	High	Questionnaire, PEFR, lavages, skin tests, blood
4	1	Four months after first *Bt* spray	Same	High	Questionnaire, PEFR, lavages, skin tests, blood
5	2	Control cohort	Onions	Low	Questionnaire, PEFR, lavages, skin tests, blood
6	3	Control cohort	Mixture of groups 1 and 2	Medium	Questionnaire, PEFR, lavages, skin tests, blood

Abbreviations: *Bt, Bacillus thuringiensis*; NA, not applicable; PEFR, peak expiratory flow rate.

Table 2. Demographic data.

Group	Activity performed	Relative exposure	Test time[a]	*n*	Male	Mean age	Female	Mean age	Atopic	Nonatopic
1	Picked *Bt*-sprayed crops	Low	Prior to	48	46	38.3	2	37	12 (25%)	36
1	Picked *Bt*-sprayed crops	High	1 month later	32	30	30	2	36	10 (31%)	22
1	Picked *Bt*-sprayed crops	High	4 months later	20	18	32	2	36	7 (35%)	13
2	Picked *Bt*-untreated crops	Low	NA	44	32	26.9	12	29	10 (23%)	34
3	Washed and packed *Bt*-sprayed and -untreated crops	Medium	NA	34	14	23.8	20	30.8	11 (32%)	23

Abbreviations: *Bt, Bacillus thuringiensis*; NA, not applicable.
[a]Relative to first exposure.

was defined as positive tests to two or more aeroallergens. Skin-prick tests to various *Btk* skin test preparations were performed on the other forearm. To exclude irritant effects, all *Btk* extracts were pretested in six nonatopic volunteers, all of whom demonstrated negative reactions. Negative (saline) and positive controls (10 mg/mL histamine phosphate base) were also used. A positive skin-prick test was defined as a wheal at least 3 mm greater than the negative control 15 min after application. At repeat visits (visits 2 and 3) all test procedures except the questionnaire were repeated.

Preparation of* Bt *extracts. Four types of *Btk* spore and two vegetative (*Btk* and *Bta*) antigens were prepared. All extracts were filter sterilized and stored in aliquots at -20°C. Javelin water-soluble pesticide extracts (J-WS) were made by mixing 10 g of dry pesticide to 100 mL phosphate buffered saline (PBS; 0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4) containing 0.4% phenol. The suspension was stirred for 48 hr at 4°C and centrifuged at 10,000*g* for 15 min. Javelin mercaptoethanol-sodium dodecyl sulfate (J-ME-SDS) spore extracts were prepared by a modification of Stelma et al. (*14*). A 20% w/v suspension of Javelin pesticide was prepared in the ME-SDS solution consisting of 0.1 M ME and 1% SDS. It was placed in a shaker water bath at 37°C for 90 min and centrifuged at 10,000*g* until the supernatant was clear. The cell pellet was resuspended in ME-SDS and the procedure was repeated. Clear supernatants were pooled and dialyzed (molecular weight cutoff of 3,500) against three changes of 0.15 M NaCl over 3 days at 4°C. Finally, the saline exchange fluid was changed to 1 L of phenolated (0.4%) PBS and dialyzed overnight. Javelin proteinase K spore extracts (J-PK) were prepared by a modification of the method described by Drobniewski and Ellar (*15*), in which 3 g Javelin was suspended in 30 mL of solution containing 0.2% SDS, 0.1% proteinase K (Sigma, St. Louis, MO) in 10 mM Tris-HCl and 1 mM EDTA, pH 9.5. This suspension was then shaken for 12 hr at 37°C, followed by centrifugation at 10,000*g*. To inhibit proteinase K activity, phenylmethylsulfonyl fluoride was added to the supernatant to a final concentration of 1 mM. The pellet was reextracted using the same procedure but with a higher concentration of proteinase K (0.5%). The two supernatants were combined and exhaustively dialyzed against isotonic saline (three changes of 4 L) and then against phenolated saline. To prepare Javelin-associated pro-δ-endotoxin (J-PROTOX), a subculture of *Btk* spores was obtained from AK Agar #2 Sporulating Agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) for 5 days at 37°C. Cells were retrieved by gentle scraping with a cell scraper and washed six times in sterile saline (0.15 M NaCl). One volume of cell-spore–cry pellet was suspended in 4 volumes of 50 mM Na_2CO_3, pH 10, sonicated at 50 W for 5 min, extracted overnight in a 37°C water bath and centrifuged at 16,000*g* for 30 min. This substrate was used to derive the alkali-solubilized inactive protoxin by a method modified from Drobniewski and Ellar (*15*). Vegetative extracts of *Btk* and *Bta* were also prepared by first culturing vegetative cells overnight on LB broth medium [Miller (Luria-Bertani) Difco Laboratories, Detroit, MI]. Two liters of an 8-hr logarithmic phase of this culture were centrifuged until the supernatant was clear. The cells were pooled in a 30-mL centrifuge tube, washed three times with sterile phenolated (0.4%) PBS, resuspended, placed in an ice bath, and sonicated for a total of 10 min at 50 W. This was done in 1-min bursts, chilling on ice between sonications. The cell suspension was centrifuged at 10,000*g* for 15 min or until the supernatant was clear. Extracts of *Bta* isolated from Agree (Novartis, Greensboro, NC) were prepared in the same manner. Extracts to the four *Btk* spore preparations were used for skin and antibody tests, whereas supernatants of *Btk* and *Bta* vegetative strains were used only for antibody assays.

Humoral IgG and IgE antibodies. Antibody assays were restricted to the J-WS and J-ME-SDS antigens because of the paucity of skin test reactions to either J-PK or J-PROTOX. Because vegetative organisms were detected in nasal washes, humoral responses to *Btk* and *Bta* vegetative antigens were also assessed. Protein estimation of all extracts was carried out by the BCA protein assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. All extracts were sterilized by passage through a 0.22-µg nitrocellulose membrane filter, checked for sterility, and dispensed in sterile vials.

An enzyme-linked immunosorbent assay (ELISA) method was used for assays of specific IgG and IgE antibodies to these *Bt* microbial antigens. Immulon 2 ELISA plates (Dynetech, Chantilly, VA) were coated with 0.1-mL 10 µg/mL protein of the respective antigen, incubated for 3 hr at 37°C, and kept overnight at room temperature. The wells were washed three times with PBS containing 0.05% Tween-80 (T-PBS) at pH 7.2. Samples of the test sera (0.1 mL, diluted 1:10) in T-PBS containing 0.1% bovine gamma globulin (T-PBS-BGG) for IgG and in T-PBS containing 1.25% egg albumin (T-PBS-OA) for IgE were added in triplicate to the microtiter plates and incubated for 90 min at 25°C, after which the wells were aspirated and washed three times with T-PBS. For the IgG assay, alkaline phosphatase conjugated goat-anti-human IgG (Sigma) was diluted 1:20,000 in T-PBS containing 0.002% bovine serum albumin. For the IgE determination the alkaline-phosphatase labeled rabbit-anti-goat IgG and goat-anti-human IgE (Kierkegard and Perry, Gaithersburg, MD) reagents were diluted 1:2,000 and 1:1,000, respectively, in T-PBS-OA. Aliquots (0.1 mL) of these reagents were added in each well and incubated for 90 min at 25°C, followed by washing three times with T-PBS, twice with water, and the addition of 0.1 mL of the developing solution, which consisted of 0.1 mg/mL *p*-nitrophenol phosphate substrate (Sigma) in diethanolamine buffer (pH. 9.6). Plates were read after 30 min on a Biotek Ceres (Winooski, VT) 900C microplate reader at an optical density of 405 nm. Blank wells were used to make a baseline measurement. IgG and IgE antibody levels were positive when the optical density (OD) was at least 3 standard deviations (SDs) greater than the mean OD of 28 and 14 control (*Bt* unexposed) sera (diluted 1–10), respectively.

Inhibition assays were performed using 0.1 mL pools of high titer IgE and IgG J-WS specific sera that were incubated at 37°C for 30 min with 0.1 mL of varying concentrations of J-WS (in micrograms). After incubation, 0.1 mL samples of these mixtures were used in the ELISA procedure, with J-WS-coated ELISA plates. Percent inhibition of antibody binding was calculated: Percent inhibition = 1 - [(OD of the inhibited sera) × (OD of noninhibited sera)] × 100.

Nasal and mouth wash samples for verification of* Btk *exposure. To verify that the bacteria recovered from cultures of the nasal/mouth lavages of the workers were in fact derived from Javelin or Agree and not from other organisms (*16*), we used a cellular and molecular genetic identification approach as a means of establishing the authenticity of exposure. Thus, we assessed both colony morphologic and staining characteristics of cultured organisms as well as their diagnostic gene content as determined by hybridization probes, using polymerase chain reaction (PCR) amplified segments of *Btk* cry 1 Ab and cry 1 Ac genes.

Nasal and mouth washes were collected from group 1 workers at specified intervals: [before spraying (visit 1), 1 month after spraying (visit 2), 4 months later (visit 3)] and from group 2 and 3 workers on separate visits. After hyperextension of the subject's head, 20 mL high grade, bacteria-free mineral water was gently infused into each naris and the nasal wash materials were collected 30 sec later in a wide-mouth sterile polyethylene container.

In addition, separate oral samples were obtained after mouth rinsing and gentle gargling of 20 mL mineral water. These samples were stored at -80°C and aliquots were used to obtain counts of microbial flora by plating on LB-agar and incubating overnight at 37°C. Isolates [individual colony-forming units (CFU)] recovered from the plate spreads were typed on the basis of colony morphology and Gram staining characteristics of the organisms. Putative *Btk* positive isolates were arranged in 96-well microtiter plates containing LB broth. These organisms were tested for the presence of *Btk* genes by a DNA–DNA hybridization procedure ("PCR Assay"). As a prestep, matching 96-well seed plates were seeded with *Bt* in 100 µL LB broth and incubated at 37°C for 6–8 hr to obtain vegetatively growing cells. The cells were lysed and DNA contents denatured by mixing with 1/10 stocks of SDS (20%) and NaOH (3N). An aliquot (10 µL) of each isolate was collected onto a nylon membrane (0.45 µm pore size) using a 96-well vacuum filtration manifold (0.45 µm pore size, 10 cm × 13.2, Zeta probe; Bio-Rad, Mississauga, Ontario, Canada). The filters were neutralized by blotting (cell side up) on 1 M Tris-HCl, air dried, and baked (*in vacuo* at 80°C). Storage was in sterile containers at room temperature with desiccant.

Analysis of the commercial* Btk *product and a field sample of that product. Most commercial *Bt* products are essentially concentrates of large-scale sporulation phase cultures of *Btk*, *Bta*, or *Bti*. The chief commercial product (Javelin) used during these investigations consisted solely of the *Btk* strain and was obtained from a local supplier as a powder. Another commercially available product (Agree) containing the *Bta* strain had also been used during the previous year. Samples of the aqueous spray formulations of Javelin were collected onsite at the time of their field application. Authentic reference strains of *B. subtilis*, *Btk*, and *Bti* were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and formulations provided as standards by the industry to Health Canada [Ottawa, Ontario, Canada (V.L.S.)]. Because the liquid *Bt* formulations were viscous, homogeneity was ensured by careful mixing and dilution aliquoting. A Class II biohood and other protective measures were used to prevent contamination. Diluents included autoclaved and filter-sterilized deionized distilled water and bacterial growth medium (LB medium; GIBCO/BRL, Burlington, Ontario, Canada). CFU per milliliter of spray formulations were deduced by spread plating of $\log_{10}$ serial dilutions (50–100 µL aliquots) on agar plates containing LB medium. Colonies of bacteria derived from the field samples as well as the aqueous mixture of commercial Javelin were examined by light microscopy after Gram staining and spore staining. The commercial products (Javelin and Agree) were also examined by scanning electron microscopy using a JSM6400 (Joel, Boston, MA) and gold-shadowed fresh mounts on aluminum foil and assessed for specific *Bt* genes using PCR and hybridization methods described below (*2*).

PCR assays, probes, and DNA hybridization assays. *Btk* and *Bta*, but not *Bti*, contain several closely related genes encoding δ-endotoxins (cry 1Ab and cry 1Ac) and 16s ribosomal genes that have been sequenced. Oligonucleotide amplimers specific for each of these genes were used for establishing the presence of *Btk* in the commercial *Btk* product, two commercial *Bta* products (Agree and Xentari; Abbott Laboratories, Abbott Park, IL) and CFU derived from them, and clinical wash samples (*2*). Direct PCR amplification of these gene sequences from the spore-containing *Btk* products was carried out using reagents and a thermal cycler (Model 9600) supplied by Perkin-Elmer (Mississauga, Ontario, Canada) (*17*). The PCR technique was adapted for use with heat-disrupted spores (95°C for 5 min) as templates by using a 50-µL reaction mixture containing *Thermus aquaticus* (Taq) DNA polymerase (2 units) and 3 mM $MgCl_2$. Digoxigenin (DIG)-labeled probes were made by using PCR products as templates (approximately 2 ng) and 10-µL 10 × DIG-dUTP nucleotide mix (Boehringer Mannheim, Laval, Quebec, Canada). Analysis of PCR products was conducted as previously described (*18*). Probe stocks were prepared by pooling several reactions of each type of DIG-labeled probe and storing at -20°C until use. Hybridizations were conducted in batches of eight using sterile multiblot trays (8.5 cm × 12.5 cm; Robbins Scientific, Toronto, Canada) according to manufacturer's recommendations, using DIG-labeled probes and membranes (Boehringer Mannheim and Bio-Rad). Photographic prints were taken of each hybridization panel using either black and white (667 Polaroid; Polaroid Corp., Cambridge, MA) or color (400 ASA) film (*2*).

Statistics. The data were analyzed as 2 × 2 contingency tables. Homogeneity and independence questions were addressed by χ^2 analysis. Changes in responses between visits of group 1 workers were analyzed using McNemar's test. Student's two-tailed *t*-test was used to compare specific IgE antibody titers before and after varying periods of exposure of group 1 workers.

Table 3. Summary of number of symptoms derived from questionnaire data.

Exposure group	Total no.	Eye[a]	Nasal[a]	Cough[b]	Dyspnea[b]	Wheeze[c]	Skin[d]	Myalgias[e]
1 (high)	48[f]	6	6	2	0	0	2	2
1 (high)	32[g]	1	4	1	0	0	5	1
2 (low)	44	2	0	0	1	0	5	3
3 (medium)	34	4	2	1	0	1	2	2

Bt, Bacillus thuringiensis.
[a]Symptoms of rhinoconjunctivitis were seasonal in 10 workers. Nasal symptoms were perennial in two workers. [b]Cough in three workers and dyspnea in one worker were present in cigarette smokers. [c]One worker complained of asthma year-round, with exacerbations after viral infections. [d]Three workers exposed to *Bt* spraying gave histories consistent with skin contact reactions after handling parsley, spinach, or celery; nine workers experienced transient hives unrelated to *Bt* exposure. [e]In all cases, myalgias were associated with musculoskeletal problems caused by physical strains during work. [f]Total number of group 1 workers prior to first spray exposure. [g]Total number of group 1 workers who were reevaluated after first spray exposure.

Table 4. Number of *Btk* positive skin-prick tests.

Visit	Time of spraying	Exposure category	*n*	*Btk* extract J-WS[a]	J-ME-SDS[b]	J-PK[c]	J-PROTOX[d]
Longitudinal study of group 1							
1a	Before	Low	48	4	1	1	0
1b[e]	Before	Low	32	3	1	1	0
2	1 month later	High	32	16*	7*	2	0
3	4 month later	High	22[f]	14*	8*	1	1
Cross-sectional study of all exposure groups							
		High	34[g]	16**	7	2	0
		Low	44	5	2	0	0
		Medium	34	5	4	0	1

Abbreviations: *Btk*, *Bacillus thuringiensis* subspecies *kurstaki*; J-ME-SDS, Javelin (Sandoz Agro, Inc., Des Plaines, IL) mercaptoethanol-sodium dodecyl sulfate; J-PK, Javelin proteinase K spore extracts; J-PROTOX, Javelin-associated pro-δ-endotoxin; J-WS, Javelin water soluble pesticide extracts.
[a]Protein = 2.5 mg/mL. [b]Protein = 1.25 mg/mL. [c]Protein = 0.31 mg/mL. [d]Protein = 0.69 mg/mL. [e]Visit 1a workers who returned on visit 2 after first spraying. These workers comprise the high-exposure group. [f]One worker not evaluated on visit 1; one worker not evaluated on visit 2. [g]Two of these workers were not evaluated prior to the first spray. *Significantly different from preexposure visit ($p < 0.05$). **Significantly different from both low and medium exposure groups ($p < 0.05$).

The longitudinal assessment of group 1 workers compared high exposure (visit 2) and continued high exposure (visit 3) to preexposure (visit 1). The cross-sectional analysis compared high (group 1, visit 2), medium (group 2), and low (group 3) exposure groups. Agreement (correlation) of response between two dependent variables was measured by the κ statistic of agreement.

Significance was predicated on $p < 0.05$.

Results

Clinical results. The questionnaire-derived data revealed that although ocular and dermatologic symptoms (12 each) were most common in the combined population, they were distributed among the three worker groups (Table 3). At least three reports of skin symptoms appeared to be due to irritant/contact dermatitis of forearms after contact at work with parsley, spinach, or celery. None of the eye symptoms or the remainder of skin symptoms could be attributed to occupational factors. A total of eight workers reported the occurrence of nonoccupational nasal symptoms. Lower respiratory symptoms were noted in four workers, none of whom could relate them to occupational exposure. Peak expiratory flow rate data were higher than 90% of predicted normal in all workers except four who were heavy smokers.

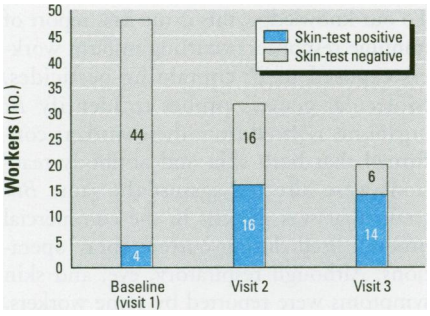

Figure 1. Skin test results in group 1 workers. At the baseline visit, 4 of 48 workers showed a positive skin test to at least one of the four *Bacillus thuringiensis* subspecies *kurstaki* test extracts. After the initial spraying (visit 2), half of the 32 workers who were reassessed exhibited positive skin tests. Skin test reactivity persisted in 14 of the 20 workers who were tested on all visits. Not included in these data were two workers evaluated during visit 2 but not visit 1; both had positive skin tests. Positive skin tests were also observed in five of seven workers who were tested for the first time in visit 3. One of two workers who were assessed in visits 1 and 3, but not 2, was also skin-test positive.

Skin test results. The prevalence of atopy in high-exposure group 1 workers was 31% as compared to 23 and 32% in the low- and medium-exposure workers (groups 2 and 3), respectively (Table 2). Skin tests results are summarized in group 1 workers before first *Bt* exposure and 1 and 4 months after repetitive exposures (Table 4) as well as in groups 2 and 3, each of which was tested on a single visit (Table 4). Most of the significant positive skin tests were elicited by aqueous extracts of the commercial J-WS and the J-ME-SDS *Btk* preparations. Positive skin-prick tests to J-PK and J-PROTOX extracts were observed in four and two workers, respectively. Relatively few positive skin tests were exhibited by workers prior to their first *Btk* exposure. However, there was a significant increase ($p < 0.05$) in the number of positive skin tests to both J-WS and J-ME-SDS extracts 1 and 4 months after workers were exposed to *Btk* spraying. Further analysis of 32 group 1 exposed workers who were tested on two occasions (baseline and 1 month after spraying) revealed that skin-prick tests converted from negative to positive in 13 and remained positive in three of four workers who were positive at baseline (Figure 1). Similarly, in 22 *Btk* exposed workers, 20 of whom were serially tested on 3 visits (baseline, 1, and 4 months after spraying), J-WS skin-prick test conversions from negative to positive were noted in 13 workers ($p < 0.01$), whereas skin test reversions from positive to negative occurred in two workers. Five of 44 group 2 low-exposed workers showed skin-prick test reactivity to the commercial J-WS extract (Table 4). Two workers in this group also reacted to the J-ME-SDS extract. Five of 34 group 3 medium-exposed workers exhibited positive responses to J-WS extract, whereas four of these also were positive to J-ME-SDS. The number of positive skin tests to J-WS in the high-exposure group was significantly increased as compared to either the low- or medium-exposed groups (Table 4; $p < 0.05$).

Possible associations between cigarette smoking, atopy, and skin-prick test reactivity to *Btk* extracts were also evaluated. A history of cigarette smoking was not a risk factor for skin sensitization to *Btk* in any of the groups. However, atopic status was significantly higher ($p < 0.001$) among skin-test-positive versus skin-test-negative workers who had less *Btk* exposure (groups 2 and 3). In contrast, atopy did not appear to play a role in increased skin-prick test sensitivity in more heavily *Btk* exposed workers (group 1).

Antibody results. Specific antibody results are summarized in Table 5. A χ^2 analysis between groups revealed that the total number of J-WS and J-ME-SDS antibody positive subjects was significantly greater ($p < 0.05$) in high-exposure workers (group 1, visit 2) than in either low- or medium-exposure workers. These data were similar for both IgG and IgE isotypes. It is apparent that some workers already had IgG antibodies to either the crude commercial J-WS or the J-ME-SDS extracts prior to the first spray operation and 1 month after the first spray. Although IgG antibodies to J-WS were not detected

Table 5. *Bacillus thuringiensis*-specific spore and vegetative humoral antibody results.[a]

Visit	Time of spraying exposure	Exposure category	*n*	Number of positive tests (total tested)[b] IgE J-WS	IgE J-ME-SDS	IgE *Btk* Veg[c]	IgE *Bta* Veg[c]	IgG J-WS	IgG J-ME-SDS	IgG *Btk* Veg[c]	IgG *Bta* Veg[c]
Longitudinal study of group 1											
1a	Before	Low	48	4 (42)	3 (42)	4 (42)	14 (42)	21 (39)	23 (39)	20 (30)	22 (30)
1b[d]	Before	Low	32	3 (26)	3 (26)	3 (26)	9 (19)	10 (20)	13 (20)	11 (20)	12 (20)
2	1 month later	High	32	12 (26)*	4 (26)	4 (26)	4 (19)	9 (20)	15 (20)	4 (20)	3 (20)
3	4 month later	High	22[e]	5 (10)	1 (8)	0 (8)	0 (8)	0 (6)	2 (6)	2 (6)	0 (6)
Cross-sectional study of all exposure groups											
		High	32	13 (31)**	6 (31)**	7 (31)	6 (23)	11 (31)[#]	14 (29)**	5 (29)	4 (29)
		Low	44	1 (37)	1 (37)	19 (37)	27 (37)	1 (36)	5 (36)	12 (36)	1 (36)
		Medium	34	1 (32)	1 (31)	14 (32)	9 (32)	6 (26)	5 (26)	7 (26)	1 (26)

Abbreviations: *Bta*, *Bacillus thuringiensis* subspecies *aizawai*; *Btk*, *Bacillus thuringiensis* subspecies *kurstaki*; IgE, immunoglobulin E; IgG, immunoglobulin G; J-ME-SDS, Javelin (Sandoz Agro., Inc., Des Plaines, IL) mercaptoethanol-sodium dodecyl sulfate; J-WS, Javelin water-soluble pesticide extracts.

[a]Controls consisted of urban, *Bt*-nonexposed subjects (14 for IgE results; 28 for IgG results). [b]The totals of tested sera vary from the number of workers seen on each visit because some subjects refused phlebotomy and some sera were no longer available at time of testing. [c]Vegetative extracts. [d]Visit 1a workers who returned on visit 2 after first spraying. These workers comprise the high exposure group. [e]One worker not evaluated on visit 1; one worker not evaluated on visit 2. *Significant as compared to group 1b prior to exposure ($p < 0.05$). **Significant as compared to low- and medium-exposure groups ($p < 0.05$). [#]Significant as compared to low exposure group ($p < 0.05$).

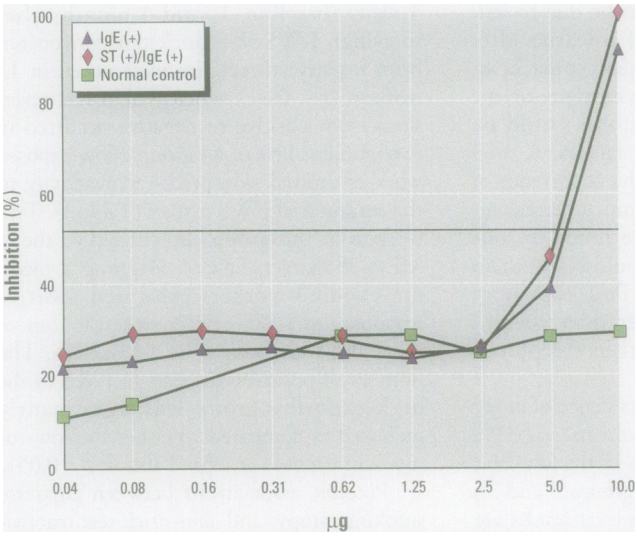

Figure 2. Mean inhibition curve of J-WS IgE antibody responses. Abbreviations: +, positive; IgE, immunoglobulin E; J-WS, Javelin (Sandoz Agro, Inc., Des Plaines, IL) water-soluble pesticide extracts; ST, skin test. Pooled sera from 11 high-titer J-WS specific IgE sera, three J-WS specific IgE sera from skin test-positive workers, and six normal control sera were preincubated with varying doses (µg) of J-WS at 37°C for 30 min. Aliquots (0.1 mL) of the samples were then used in the enzyme-linked immunosorbent assay and percent inhibition was calculated as described in "Methods."

in sera collected on visit 3, this may be partially artifactual because only six sera were available on this occasion and of these, IgG antibody to J-WS had only been demonstrated in two sera. Specific IgE antibody levels were present in a few workers before the first spray, increased 1 month later, and tended to persist 4 months later. Only five workers in group 2 exhibited either IgE or IgG antibodies to J-WS and J-ME-SDS. One worker in group 3 showed J-WS-specific IgE antibodies, whereas specific IgG antibodies to J-WS were detected in six workers. None of the control nonexposed subjects had specific IgG or IgE isotypic antibodies. The specificity of J-WS IgE ELISA results was demonstrated by an antigen inhibition assay on pooled positive sera as compared to normal control sera (Figure 2). Similar data (not shown) were obtained for J-WS-specific IgG antibodies.

A subanalysis of antibody results in group 1 workers with one or more positive skin-prick tests to *Btk* skin-test extracts revealed that approximately 36% of them had specific IgG antibodies prior to spraying, whereas specific IgE antibodies were undetectable. One month after spraying, there was no appreciable change in IgG results but significant J-WS specific IgE titers were demonstrated in 5 of 16 skin-test-positive high-exposure workers. Although there was not a correlation between skin test positivity and the cut-off level of J-WS-specific IgE [≥ mean + 3 SD (0.18) of a pool of 14 nonexposed urban control sera], there was a definite trend to rising specific IgE antibody titers 1 month after exposure. This reached significance in 10 preexposure and 4-month postexposure paired sera [prior exposure: mean OD, 0.08 ± 0.01 standard error of the mean (SEM); postexposure: mean OD, 0.22 ± 0.07 SEM, p = 0.05; 14 nonexposed urban controls; mean OD 0.12 ± 0.01 SEM].

To further assess immunologic response to *Bt* antigens to which workers were exposed in current and previous spraying, available sera of all groups were also analyzed for both *Btk* and *Bta* vegetative specific IgG and IgE antibodies (Table 5). Elevated levels of specific IgG antibodies to *Btk* vegetative antigens were detected in 17, 30, and 27% of high, low, and medium exposure groups, respectively. Specific *Btk* vegetative IgE antibodies were also present in all three groups (23% in group 1 after exposure; 51% in group 2; and 44% in group 3). Table 5 also demonstrates the occurrence of *Bta*-specific IgG and IgE antibodies in all worker groups except that the IgG isotype was rare in groups 2 and 3.

Recovery of* Btk *organisms from oral and nasal samples from workers. Verification of *Btk* in samples recovered from the current *Btk* spray operations is demonstrated in Figure 3. Both agarose (1.5%) gel electrophoresis analysis (Figure 3A) and hybridized blot analysis (Figure 3B) revealed the presence of genes encoding cry 1 Ab, cry 1 Ac δ-endotoxins, and 16 S ribosomal RNA in both the Agree and Javelin strains of *Bt* to which these workers were previously and currently exposed.

Culture-positive (by morphology and Gram stains) *Btk* colonies were obtained in only four mouth lavage samples in group 1 and in no samples from groups 2 and 3. However, the recovery yield of *Btk* organisms from nasal irrigation was much greater (Table 6). Thus, 1 month after exposure to *Btk* spray, the majority (66%) of nasal lavage cultures of group 1 workers were positive for *Btk*, whereas only two positive nasal lavage cultures were detected in the same workers prior to spray exposure. As shown in Figure 3C, the organisms recovered from these nasal lavage cultures were genetically identical to those found in the commercial spray as well as to a reference ATCC *Btk* specimen, two other commercial sprays (Agree and Xentari) containing *Bta* but not the ATCC *Bti* reference. Cultures from nasal wash specimens of five group 1 workers continued to be positive throughout the remainder of the spraying season. Positive nasal lavage *Btk* cultures were also observed in eight workers in group 2 and nine workers in group 3. Although specific *Bt* identification by nasal lavage occurred with a greater frequency in high as compared to low- or medium-exposure groups ($p < 0.05$), correlations between positive skin tests and/or antibodies were not demonstrated.

Discussion

To our knowledge, this is the first report of immune responses occurring in farm workers exposed to *Bt*-containing pesticides. Molecular genetic probes to identify *Bt* organisms isolated from these workers confirmed that both skin and antibody reactions were directed against the same *Btk* strain that was present in the commercial product used during current spray operations. Although respiratory, eye, and skin symptoms were reported by some workers, none of these symptoms could be attributed to previously established case definitions of occupationally related disease. The few ventilatory abnormalities detected by peak expiratory flow rate testing were found in heavy smokers. Nevertheless, the lack of clinical disease in this cross-sectional survey should be interpreted with caution because of the healthy worker effect, which might be more prevalent among migrant farm workers who, upon associating clinical symptoms with a particular crop or farm job, would likely seek employment in a different agricultural area. Moreover, clinical symptoms would not be anticipated unless there was repeated long-term exposure and more vigorous antibody responses to these organisms.

The survey for possible skin sensitivity was performed with aqueous extracts of a commercial spray product and three antigens derived from sporulation cultures of *Btk*, including one containing the *Btk* crystalloid δ-endotoxin. The majority of positive skin-prick tests to *Btk* occurred in workers who had a higher degree of exposure. Moreover, the number of positive skin-prick tests to both J-WS and J-ME-SDS extracts increased 1 month after exposure and persisted for 4 months after repetitive exposure to Javelin spray. Although it is possible that some skin test responses to J-WS could have been induced by non-*Btk*

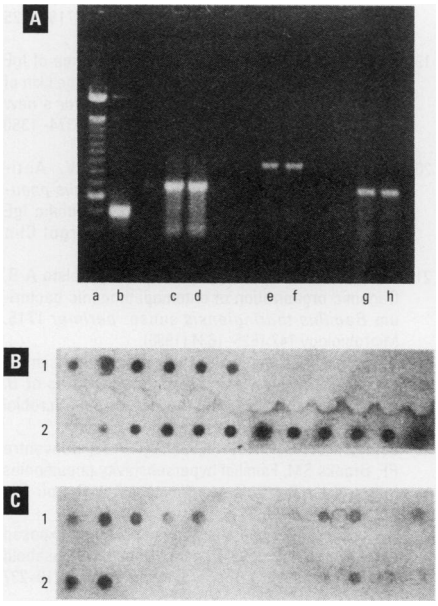

Figure 3. Verification of *Btk* in samples recovered from *Bacillus thuringiensis* spray operations. Abbreviations: ATCC, American Type Cell Collection (Rockville, MD); cry, toxin crystal; *Btk*, *Bacillus thuringiensis* subspecies *kurstaki*; PCR, polymerase chain reaction. (*A*) Agarose (1.5%) gel electrophoresis analysis of PCR products amplified from control template (50 ng lambda DNA) mixed with Javelin (Sandoz Agro, Inc., Des Plaines, IL; lane b); Javelin alone (lanes d, f, h); or reference *Btk* product alone (Xentari, Abbott Laboratories, Abbot Park, IL; lanes c, e, g), all diluted to give 10^4 spores (colony-forming units) final; oligonucleotide primers (20 pM of each) specified lambda (lane b) and the genes encoding cry 1 Ac (lanes c, d), cry 1 Ab (lanes e, f) and 16s ribosomal RNA (lanes g, h). Lane a contains DNA size markers (100 base-pair ladder). (*B*) and (*C*) show panels (12 dots per row) of dot-blot DNA hybridizations using a pool of PCR amplified, digoxigenin-labeled, cry 1 Ab and cry 1 Ac gene probes [see (*A*) and "Methods"]. (*B*) Rows 1 and 2 show positive results (dots 1–6) for *Btk* ATCC strains, negative results (dots 7–12) for ATCC strains of *Bacillus thuringiensis* subspecies *israelensis* and positive results (row 2) for three products: Agree (Novartis, Greensboro, NC; dots 1–3), Xentari (dots 4–6), Javelin (dots 7–9), and Javelin recovered from spray tank (dots 10–12). (*C*) *Btk* detection results of individual bacterial colonies recovered from nasal samples of three workers immediately after their exposure to Javelin spray. In this technique the appearance of weakly staining dots is also considered positive.

fermentation media contaminants in the extract of the commercial product, many of the workers who demonstrated positive tests to J-WS also reacted to the J-ME-SDS antigen derived from pure *Btk* spore cultures. Skin reactivity to the J-PK spore extract was rare (four workers). Only two workers among all three groups demonstrated a positive skin-prick test to J-PROTOX, the *Btk* spore extract containing the pro-δ-endotoxin active component.

Table 6. Results of nasal lavage DNA hybridization identifying exposure to *Bacillus thuringiensis* subspecies *kurstaki* among worker groups.

Visit	Exposure category	No. tested	DNA positive	Mean CFU/20 mL
Longitudinal study of group 1				
1a	Low	47	3	200
1b[a]	Low	31	2	100
2	High	31	21*	532
3	High	22[b]	6	5
Cross-sectional study of all exposure groups				
	High	32	22**	540
	Low	44	8	23
	Medium	34	9	20

CFU, colony-forming units.
[a]Visit 1a workers who returned on visit 2 after first spraying; these workers comprise the high-exposure group. [b]Nasal lavage of two additional workers tested only during visit 3. *Significantly different from preexposure visit 1b ($p < 0.05$). **Significantly different from low and medium exposure groups ($p < 0.05$).

Intergroup comparisons between the prevalence of IgG and IgE immune responses indicate that exposure to *Btk* spray may lead to allergic sensitization, as indexed by both positive skin tests and specific IgE antibodies, induction of IgG antibodies, or both. Thus, a significant number of workers had IgG antibodies before the first spray operation of the season, perhaps a reflection of their exposure to *Btk* in previous years. In contrast, the increase of IgE antibody 1 month after spraying in group 1 workers is consistent with the anamnestic response induced by exposure to classical allergens. This was especially notable in *Btk* skin-test-positive workers who exhibited a rise in IgE antibodies without concomitant changes in IgG antibodies after spray exposure. Paradoxically, skin sensitization to *Btk* was associated with atopy only in the group of less heavily exposed atopic workers. This might suggest that heavy bacterial exposure could induce an IgE-mediated response even in nonsusceptible populations, whereas lower levels of bacterial impaction within the respiratory tract could suffice to affect atopic individuals. Despite the fact that commercial spray products contain only trace amounts of vegetative organisms (*2*), detection of *Btk* vegetative-specific IgG and IgE antibodies in many of these workers suggests that vegetative forms had presumably germinated from *Bt* spores lodged in the upper respiratory tract and were shed into nasal wash specimens. *Btk* spores germinate and produce vegetative cells at pH and temperature preferences comparable to mammalian conditions (*2,17*). This indicates that allergenic effects of *Btk* in humans could be due in part to vegetative-derived allergens. The presence of IgG and IgE antibodies to vegetative *Bta* extracts could either reflect the known previous exposure of these workers to *Bta* or the presence of cross-reactive epitopes between *Btk* and *Bta* vegetative extracts.

Although occupationally related clinical diseases were not observed in this cross-sectional survey, the fact that skin and serologic tests of immediate hypersensitivity developed in some workers indicates that adverse IgE mediated health effects could develop if repetitive exposures continue in some of these workers. Longitudinal surveillance studies will be necessary to establish whether this would occur. These results also suggest that future large-scale urban spraying of *Bt* pesticides may not be innocuous and may require more direct health monitoring and surveillance.

In addition to the implication that skin sensitization to *Bt* in pesticides could be a precursor of clinical IgE-mediated diseases, several aspects of this investigation may be relevant to other current health issues: immediate hypersensitivity induced by bacteria and transgenic foods engineered to incorporate pesticidal genes in their genomes. First, because skin sensitivity to spore and vegetative components of a nonpathogenic species of *Bacillus* was clearly demonstrated, future awareness about the allergenic potential of environmental bacteria should be increased, even though this phenomenon has been recognized for relatively few such organisms (e.g., *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*) (*19,20*). There is presently strong evidence of a close molecular genetic relatedness between *Bt* subspecies and the *B. cereus* food pathogen that would support this call for caution (*21,22*). Further, in the case of the *Bacillus* genus, the possibility of cross-allergenic epitopes in an unrelated species such as *B. subtilis* should be appreciated because this organism or its products may occur in both occupational and nonoccupational environments (*23,24*). Conversely, results of this investigation should partially allay recent concerns about the occurrence of possible adverse health effects in consumers after exposure to transgenic foods (*25,26*). Because reactivity to the *Btk* pro-δ-endotoxin was only encountered in 2 of 123 workers sensitized by the respiratory route, it is unlikely that consumers would develop allergic sensitivity after oral exposure to transgenic foods (e.g., tomatoes, potatoes) that currently contain the gene encoding this protein. However, future clinical assessment of this possibility is now feasible because of the availability of reliable *Bt* skin and serologic reagents developed during the course of this investigation.

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